Of course, the particular constituents and concentrations of those constituents can differ between in vitro and in vivo assays as well as between different mammals whose cells are to be assayed. Such constituents and concentrations can be readily determined by skilled workers. It is to be further understood that a previously described fusion protein that includes an immunologically active portion of a mycobacterial 65KD cell wall protein-a antigen can also be used to the exclusion of a peptide or polymer thereof as the antigen. Thus, the antigen of the kit can more broadly be described as a mycobacterial antigen.

A useful peptide corresponds substantially in sequence to a sequence of either the 540 or the 517 proteins discussed previously. Substantial correspondence of peptide sequences can be determined in a number of ways.

Of course, two peptides having identical sequences correspond substantially, as do to peptides that share identical sequences but also contain one or more further sequences. Similarly, two sequences that differ by conservative substitutions such as isoleucine for leucine or valine, asparatic acid for glutamic acid, asparagine for glutamine, arginine for lysine, serine for threonine, phenylalanine for tryptophan and tyrosine for phenylalanine, also correspond substantially.

Two sequences can also correspond substantially when antibodies raised to one immunoreact with another. For example, the particular peptides disclosed hereinafter can be used to raise antibodies that immunoreact with the 65KD (540) protein, and consequently, those peptides correspond substantially in sequence to the sequence of the 65KD protein.

Biochemical evidence from immunoassay and from analogy with conserved protein-protein interaction in solved X-ray crystallographic structures with differing sequences such as in the dimer contacts of oligomeric enzymes indicates that the conservation of protein-protein recognition does not require a strict conservation of sequence, for relatedness. Whereas single amino acid residue changes may affect such recognition to a wide degree depending upon the nature of the substitution, in general terms the relatedness and thus substantial correspondence of two differing amino acid sequences with respect to protein-protein (and antigenic and/or immunogenic) recognition can be expressed in terms of seven basic amino acid residue parameters:

(1) hydrophobicity;
(2) evolutionary occurrence of changes in known sequences;
(3) size of side chain;
(4) charge and polarity;
(5) preference for turned secondary structure;
(6) preference for beta strand secondary structure; and
(7) preference for helical secondary structure.

To define the degree of sequence identity relevant to antigenic and/or immunogenic recognition, and thus substantial correspondence of peptide variants, a consensus matrix based upon the above seven criteria can also be used to assign numerical values for each amino acid residue pair in the sequences being considered for substantial correspondence. For the purposes of the present invention, a consensus matrix developed by Dr.

Elizabeth Getzoff and Dr. John Tainer of the Scripps Clinic and Research Foundation of La Jolla, CA can be used. That consensus matrix is as follows, wherein the individual amino acid residues are designated by a one-letter code in the interests of conciseness:

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	7	-5	-1	-2	0	0	-1	2	-1	0	1	-2	2	-1	0	0	0	-3	-3	1
R	-5	10	0	-1	-3	2	-1	-5	5	-4	-4	5	-3	-2	-3	0	0	-1	-1	-4
N	-1	0	6	3	1	3	0	1	3	-2	-2	2	-1	-3	1	4	2	-3	0	-2
D	-2	-1	3	7	-2	1	4	0	0	-3	-3	0	-2	-4	0	1	0	-5	-2	-3
C	0	-3	1	-2	7	1	-2	1	0	0	0	-2	0	0	0	3	4	-2	2	0
Q	0	2	3	1	1	6	2	-1	4	0	0	2	0	0	0	1	3	-1	0	0
E	-1	-1	0	4	-2	2	7	-3	1	-3	-2	0	-1	-3	-2	0	0	-5	-3	-3
G	2	-5	1	0	1	-1	-3	8	-2	-3	-3	-2	-2	-5	2	3	1	-6	-2	-2
H	-1	5	3	0	0	4	1	-2	8	-1	0	4	0	0	0	1	2	0	2	-1
I	0	-4	-2	-3	0	0	-3	-3	-1	5	4	-3	2	2	-2	-2	0	0	0	4
L	1	-4	-2	-3	0	0	-2	-3	0	4	6	-2	4	3	-1	-2	0	1	0	3
K	-2	5	2	0	-2	2	0	-2	4	-3	-2	8	-1	-3	-1	0	0	-4	-2	-3
M	2	-3	-1	-2	0	0	-1	-2	0	2	4	-1	6	2	0	-1	0	0	-1	2
F	-1	-2	-3	-4	0	0	-3	-5	0	2	3	-3	2	7	-2	-3	0	4	3	2
P	0	-3	1	0	0	0	-2	2	0	-2	-1	-1	0	-2	7	2	1	-4	-1	-1
S	0	0	4	1	3	1	0	3	1	-2	-2	0	-1	-3	2	5	3	-3	0	-1
T	0	0	2	0	4	3	0	1	2	0	0	0	0	0	1	3	6	-2	1	0
W	-3	-1	-3	-5	-2	-1	-5	-6	0	0	1	-4	0	4	-4	-3	-2	9	2	0
Y	-3	-1	0	-2	2	0	-3	-2	2	0	0	-2	-1	3	-1	0	1	2	8	0
V	1	-4	-2	-3	0	0	-3	-2	-1	4	3	-3	2	2	-1	-1	0	0	0	5

Sequence comparison using the foregoing consensus matrix involves the determination of all possible alignments and the subsequent scoring of

these alignments by the matrix. Two sequences are then aligned by computing the maximum match score from the consensus matrix. An alignment score in standard deviation units can be determined by taking the difference between the maximum matched score and the average maximum matched score for random permutation of the two sequences, and then dividing by the standard deviation of the random score.

For the present purposes, a consensus matrix score greater than three standard deviations (approximately an average value of about 3 per residue) shows significant relatedness at a confidence level of more than 99.7%. This is a restrictive criterion since it gives a frequency of 0.00% for all 5-residue peptides and 0.001% for all 13-residue peptides occurring in 2222 known protein sequences. Similarly, a consensus matrix score greater than two standard deviations (approximately an average value of about 2 per residue) shows substantial correspondence to be significant at a confidence level of more than 95.4%.

To determine substantial correspondence for the purposes of the present invention, the consensus matrix score is calculated by ascertaining the matrix value for each aligned amino acid residue pair under consideration, and then summing the individual values for each such pair. The obtained sum is then compared against the number of standard deviations signifying the desired confidence level. If the obtained sum is greater than the product of the selected number of standard deviations times the number of amino acid residue pairs under consideration, then the amino acid residue sequences being compared correspond substantially to the indicated confidence level.

For example, to ascertain the substantial correspondence of the amino acid residue sequences

-Lys-Trp-Phe-Cys-Gly-

and

-Arg-Ile-Phe-Cys-Gly-

the consensus matrix yields the following values

	Value
-Lys- & -Arg- or K & R	5
-Trp- & -Ile- or W & I	0
-Phe- & -Phe- or F & F	7
-Cys- & -Cys- or C & C	7
-Gly- & -Gly- or G & G	8
Total	27

For substantial correspondence at the 99.7% confidence level, the consensus matrix score must exceed the number of amino acid residue pairs under consideration times 3; i.e., 5x3 or 15. Inasmuch as 27 is greater than 15, substantial correspondence is indeed present for the above two peptide sequences.

For the purposes of the present invention, substantial correspondence among peptides within the scope of the invention preferably is present at least to about 95% confidence level, and more preferably to at least about 99% confidence level.

A DNA sequence can correspond substantially to another DNA sequence if both sequences contain sequences of fifteen bases that are in phase and identical, or bases that are not identical but code for an identical sequence of amino acid residues, or code for amino acid residue sequences that correspond substantially. Thus, amino acid residue sequences that correspond substantially are encoded by DNA sequences that correspond substantially.

It is to be noted that two or more peptide sequences can substantially correspond as determined by one or both of the latter two definitions and still exhibit different immunoreactivities with antibodies raised to the intact protein as is found in nature or with T cells stimulated by such natural proteins. An example of this phenomenon is discussed hereinafter.

In addition to the specific peptides disclosed in Table 2, hereinbefore, further peptides that correspond in sequence to a portion of the 540 protein sequence are also useful herein. A list of those peptides is provided in Table 4, below.

Table 4

Peptides

Peptide Number	Residues[1]	Sequence[2]
1	1-15	M A K T I A V D E E A R R G L
2	11-25	A R R G L E R G L N A L A D A
3	21-35	A L A D A V K V T L G P K G R
4	31-45	G P K G R N V V L E K K W G A
5	41-55	K K W G A P T I T N D G V S I
6	51-65	D G V S I A K E I E L E D P Y
7	61-75	L E D P Y E K I G A E L V K E

8	71-85	E L V K E V A K K T D D V A G
9	81-95	D D V A G D G T T T A T V L A
10	91-105	A T V L A Q A L V R E G L R N
11	101-115	E G L R N V A A G A N P L G L
12	111-125	N P L G L K R G I E K A V E K
13	121-135	K A V E K V T E T L L K G A K
14	131-145	L K G A K E V E T K E Q I A A
15	141-155	E Q I A A T A A I S A G D Q S
16	151-165	A G D Q S I G D L I A E A M D
17	161-175	A E A M D K V G N E G V I T V
18	171-185	G V I T V E E S N T F G L Q L
19	181-195	F G L Q L E L T E G M R F D K
20	191-205	M R F D K G Y I S G Y F V T D
21	201-215	Y F V T D P E R Q E A V L E D
22	211-225	A V L E D P Y I L L V S S K V
23	219-233	L L V S S K V S T V K D L L P
24	231-245	L L P L L E K V I G A G K P L
25	241-255	A G K P L L I I A E D V E G E

	26	251-265	D V E G E A L S T L V V N K I
	27	261-275	V V N K I R G T F K S V A V K
5	28	271-285	S V A V K A F G F G D R R K A
	29	281-295	D R R K A M L Q D N A I L T G
	30	291-305	A I L T G G Q V I S E E V G L
10	31	301-315	E E V G L T L E N A D L S L L
	32	311-325	D L S L L G K A R K V V V T K
15	33	321-335	V V V T K D E T T I V E G A G
	34	331-345	V E G A G D T D A I A G R V A
	35	341-355	A G R V A Q I R Q E I E N S D
20	36	351-365	I E N S D S D Y D R E K L Q E
	37	361-375	E K L Q E R L A K L A G G V A
25	38	371-385	A G G V A V I K A G A A T E V
	39	381-395	A A T E V E L K E R K H R I E
	40	391-405	K H R I E D A V R N A K A A V
30	41	401-415	A K A A V E E G I V A G G G V
	42	411-425	A G G G V T L L Q A A P T L D
35	43	421-435	A P T L D E L K L E G D E A T

44	431-445	G D E A T G A N I V K V A L E
45	441-455	K V A L E A P L K Q I A F N S
46	451-465	I A F N S G L E P G V V A E K
47	461-475	V V A E K V R N L P A G H G L
48	471-485	A G H G L N A Q T G V Y E D L
49	481-495	V Y E D L L A A G V A D P V K
50	491-505	A D P V K V T R S A L Q N A A
51	501-515	L Q N A A S I A G L F L T T E
52	511-525	F L T T E A V V A D K P E K E
53	521-535	K P E K E K A S V P G G G D M
54	526-540	K A S V P G G G D M G G M D F

[1,4]See Notes 1 and 2 of Table 2.

Preferred peptides for use in the previously described assay for the presence of mycobacterially-immune mononuclear cells are those that are numbered as follows, wherein the numbers are those shown in one or both of Tables 2 and 4, and whose sequence positions in the 540 protein are given in parentheses: Peptide 22 (211-225); Peptide 23 (219-233); Peptide 24 (231-245); Peptide 30 (291-305); Peptide 46 (451-465); Peptide 58 (11-28); Peptide 59 (66-79); Peptide 60 (114-130); and Peptide 62 (394-408).

Several proliferative assays were conducted using the peptides of the invention. Results of those studied are shown and discussed below.

One study was carried out using pooled peripheral blood mononuclear cells (PBMC) from M. bovis BCG-vaccinated humans. The details of this study are described in the Materials and Methods Section. Briefly, PBMC were isolated and seeded into culture plate wells. Such PBMC populations contain their own endogenous antigen-presenting or feeder cells. A peptide of the invention was added as antigen at either 0.1 microgram per milliliter (ug/ml), 1 ug/ml or 10 ug/ml of culture. The antigen/cell culture mixture was maintained for a time period of six days, at which time, radiolabeled thymidine was admixed. The cultures were harvested eighteen hours later and the thymidine incorporation was measured in a liquid scintillation counter. The results of this study are shown in Table 5, below.

Table 5

Protein S40

Peptide-Induced PBMC Proliferation[1]

Peptide Number[2]	Residue Positions[3]	Proliferation Response[4]
10	91-105	-
21	201-215	-
22	211-225	++
24	231-245	++
25	241-255	-
30	291-305	++
35	341-355	-
43	421-435	-

46	451-465	+++
47	461-475	-
48	471-485	-
49	481-495	-
53	521-535	-
54	526-540	-
58*	11- 28	++
59*	66- 79	++
60*	114-130	+++
61*	154-172	-
23*	219-233	+++
62*	394-408	+++
63*	494-508	-

[1] Proliferation as measured by incorporation of [^{3}H]-thymidine in counts per minute (cpm).

[2] Peptide number as shown in Tables 2 and 4.

[3] Peptide residue sequence positions as shown in Tables 2 and 4 and in Figure 2.

[4] Proliferative response reported at the optimal peptide concentration is represented as follows: "+++" = 10,000-40,000 cpm; "++" 2000-10,000 cpm; or "-" = 300-700 cpm. Proliferation in the absence of peptide antigen was 421±37 cpm, and was 82,857 ±2,913 cpm in the presence of an extract of M. tuberculosis. Standard deviations did not exceed 15 percent in any of the triplicate measurements.

* Peptides predicted to form amphiphilic helices.

The above results indicate that nine of the twenty petides assayed elicited a strong proliferative response. Thus, nine regions of the 540 protein were identified as human T cell antigens.

Seven regions of the 540 protein were predicted by computer-assisted analysis to form amphiphilic helices. Regions that can form amphiphilic helices appear to have a higher probability of being recognized by T cells. Berzofsky, (1985) Science, 229:932-940. However, only five of those seven peptides provided a proliferative response. This indicates that amphiphilicity is neither sufficient nor necessary for a peptide to interact with T cells.

In further studies with PBMC from individual BCG-immunized humans, an influence of HLA type was noted on reactivity. Thus, lymphocytes from two persons with the HLA-DR4 allele reacted with Peptide 62 (positions 364-408) but not with Peptide 30 (positions 291-305), whereas cells from three persons with the HLA-DR1 allele reacted with Peptide 30 but not with Peptide 62.

The above results indicate a genetic, HLA restriction on the proliferation response. Thus, a mixture of preferred peptides or their polymers is preferred when assaying an out bred population such as humans so that false negative responses can be minimized.

Another proliferation study was carried out with sixteen of the above peptides. The proliferating cells here were either one of two T cell clones or a polyclonal T cell line. One T cell clone came from a tuberculosis patient (AH) and is designated K8AH. The second T cell clone was obtained from a person (JM) vaccinated with heat-killed M. leprae and is referred to as A7JM. The polyclonal T cell line (JM) was also obtained from the cells of JM that were initially stimulated with M. bovis BCG, stored frozen and thereafter stimulated with a recombinant 65KD protein from

M. tuberculosis (Östung et al., (1987) J. Immunol., 138:927-931].

Proliferation was again assayed by the [^{3}H]-thymidine incorporation method. Here, autologous PBMC irradiated to inhibit proliferation but sufficiently viable to act as antigen-presenting cells were added to the cultures of both isolated T cell clones and the cell line along with a mycobacterial antigen. After three days of maintenance, the cultures were pulsed with the radiolabel for four hours, harvested and then counted.

The details of this study are provided in the Materials and Methods Section. The results are shown in Table 6, below.

Table 6

Protein 540
Peptide-Induced Proliferation[1]

Peptide[2] Number	Proliferative Response[3] KSAH	A7JM	JM
59*	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
10	0.4 ± 0.2	0.1 ± 0	0.2 ± 0
21	0.3 ± 0	0.1 ± 0	0.2 ± 1
22	0.5 ± 0	14.0 ± 2.0	88.2 ± 15.8
23*	0.2 ± 0.1	0.1 ± 0.1	9.0 ± 2.8
24	7.4 ± 0.3	0.1 ± 0	4.1 ± 0.1
25	0.9 ± 0.2	0.1 ± 0	0.3 ± 0.2
30	0.4 ± 0.1	0.1 ± 0	0.7 ± 0.2
35	0.4 ± 0.1	0.4 ± 0	0.3 ± 0.1
52*	0.4 ± 0.3	0.2 ± 0	0.3 ± 0.1
43	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
46	0.5 ± 0.1	0.5 ± 0	11.4 ± 0.2

49	0.5 ± 0.1	0 ± 0	0.4 ± 0.3
53	0.1 ± 0	0.2 ± 0.1	0.2 ± 0.1
54	0.4 ± 0.2	0.2 ± 0.1	0.9 ± 0.1
63	0.4 ± 0	0.2 - 0.1	0.4 ± 0.1
-Ag[4]	0.5 ± 0.2	0.3 ± 0.2	0.4 ± 0.2
M. tuberculosis rec. 65KD[5]	4.3 + 0.5	12.3 + 1.9	30.1 + 2.6
M. tuberculosis[5]	8.5 + 1.6	11.5 + 1.5	118.7 + 5.4
M. bovis BCG[5]	9.4 + 0.7	21.6 + 2.3	183.4 + 19.0
M. leprae[5]	0.5 ± 0.1	24.2 + 2.0	119.7 + 15.5

[1,2] See notes of Table 5.

[3] Proliferative responses, in cpm x 10^{-3} ± standard derviation for two or three replicate studies using 10 ug/ml of each peptide. Positive values are underlined.

[4] Response in the absence of antigen.

[5] Affinity-purified recombinant M. tuberculosis 65 KD protein at 50 ug/ml and whole mycobacteria at 20 ug/ml.

The results shown in Table 6 illustrate the clonal specificity for antigens of the screened peptides. Thus, T cell clone K8AH, specific to the M. tuberculosis complex [Oftung et al., (1987) J. Immunol., 138:927-931] exhibited a proliferative response upon stimulation with an inoculum containing Peptide 24 (231-245). The T cell clone A7JM, which shows cross-reactivity to M. tuberculosis and M. leprae, responded to stimulation by admixture and contact with an inoculum containing a different segment of the 65KD (540 protein) represented by Peptide 22 (211-225), but not to inocula containing the flanking and overlapping Peptides 21 (201-215) and 23 (219-233).

The JM polyclonal T cell line also proliferated in response to contacting with inocula containing Peptides 24 and 22. That cell line also showed a significant proliferation in response to admixture and contact with an inoculum containing Peptide 23, whose sequence overlaps both of those sequences of Peptides 24 and 22, and that was predicted to form an amphiphilic helix. Contacting the polyclonal T cell line with an inoculum containing Peptide 46 (positions 451-465) also provided significant stimulation.

The genomic sequence of the 65KD protein of M. leprae and the putative translation product of that gene have been published. [Mehra et al., (1986) Proc. Natl. Acad. Sci. USA, 83:7013-7017.] A comparison of the 65KD protein amino acid residue sequences from M. leprae and M. tuberculosis shows the two sequences to be very similar, with only a relatively few different residues between them.

T cell clone A7JM had previously been shown to proliferate in response to stimulation by both whole M. leprae and whole M. tuberculosis. [Mustafa et al., (1986) Lepr. Rev. Suppl., 2:123-130.] Consistent with those findings, clone A7JM also proliferated when admixed and contacted with an inoculum containing Peptide 64, below, whose sequence differed from that of Peptide 22 by the conservative change of the residue at position 215 from glutamic acid of Peptide 22 to aspartic acid.

(22) A V L E D P Y I L L V S S K V
(64) A V L E E P Y I L L V S S K V.

T cell clone K8AH is able to discriminate between M. tuberculois and M. leprae presented for stimulation as whole bacilli, and was similarly able

to exhibit the same discrimination at the peptide level. Thus, the M. tuberculosis-related Peptide 24 (231-245) could be used to stimulate clone K8AH, whereas contact with an inoculum containing Peptide 65, below, having the analogous M. leprae sequence did not stimulate that clone to proliferate. Inocula containing Peptide 65 also did not stimulate clone A7JM or polyclonal cell line JM.

(24) L L P L L E K V I G A G K P L
(65) L L P L L E K V I Q A G K S L.

As can be seen from a comparison of the above sequences of Peptides 24 and 65, those peptides differ by the substitution of two residues near their carboxy-termini. The glycine (G) at position 240 of Peptide 24 is substituted as a glutamine (Q) in Peptide 65, and the proline (P) at position 244 of Peptide 24 is substituted as a serine (S) in Peptide 65.

Thus, recognition of Peptide 24 by clone K8AJ must be influenced by one or both of glycine-240 and proline-244. Interestingly, an inoculum containing Peptide 25 (241-255), which contains proline-244, did not cause stimulation of clone K8AH cells when admixed and contacted with those cells.

A blocking study was carried out to determine whether an inoculum containing Peptide 65 could inhibit the stimulatory response caused by Peptide 24 on cells of T cell clone K8AH. Those studies showed that the M. leprae-related Peptide 65 could not block the response induced by the M. tuberculosis-related Peptide 24. This finding again implies the criticality of one or both of the residues at positions 240 and 244 of Peptide 24.

Further stimulation studies were carried out using M. leprae-related and M. tuberculosis pepides and the before-mentioned T cell clones and cell line. An inoculum containing Peptide 23 caused stimulation of the polyclonal cell line. The sequence of that peptide is identical in both M. leprae and M. tuberculosis. (See also Table 6.)

In addition, two M. leprae-related Peptides, 64 and 66, that each contain a single amino acid residue substitution as compared to their analogous M. tuberculosis-related Peptides, 22 and 46, respectively, also were capable of eliciting stimulation of M. leprae-immune cell line JM when inocula containing one or the other were admixed and contacted with those cells. Neither M. leprae-related Peptide 64 nor Peptide 66 stimulated cells at T cell clone K8AH. The sequences of Peptide 66 and of its analogous Peptide 46 are shown below.

(46) I A F N S G L E P G V V A E K
(66) I A F N S G M E P G V V A E K.

Each of Peptides 64, 65 and 66 corresponds substantially to Peptide 22, 24 and 46, respectively. That substantial correspondence notwithstanding, the results above illustrate that there can be differences in reactivities of such peptides at the T cell level.

That different reactivities in T cell stimulation were found for substantially corresponding peptides that differed in sequence is not particularly surprising in view of the type of interaction thought to be involved in T cell stimulation by an antigen as compared to an antigen-antibody interaction.

Thus, an antigen-antibody interaction is usally considered to be a relatively simple ligand-receptor interaction in which substitutions of polar for polar (e.g., glutamic for aspartic in peptides 22 and 66) or apolar for apolar of about the same size (e.g., leucine for methionine in Peptides 46 and 67) typically are not of great consequence. Indeed, it has been shown that for some influenza-related 13-residue peptides, drastic substitutions can occur with little differences being noted in binding by a monoclonal antibody raised to the parent peptide. See, for example U.S Patent No. 4,631,211.

T cell stimulation, on the otherhand, is thought to resemble a sandwich in which the T cell and the antigen-presenting or feeder cell are the bread and the antigen is the filling. Thus, a peptide antigen must bind to two receptors, one on the T cell, and the other, believed to be one or more proteins of the major histocompatibility complex (MCH), on the feeder cell. In addition, the binding between antigen and each of the T cell and feeder cell receptors is thought to be weaker than is the usual antigen-antibody binding. It was not therefore surprising that the glycine to glutamine and proline to serine substitutions between Peptides 24 and 65 resulted in differences in T cell stimulation.

As noted previously, T cell stimulation can be manifest in a number of manners. The previous discussion has centered primarily on <u>in vitro</u> and <u>in vivo</u> proliferation assays. The results discussed below using T cell clones K8AH and A7JM illustrate further manisfestations of T cell stimulation, and manners in which such stimulation can be determined.

Standard assays for secretion of IL-2, granulocyte macrophage-colony stimulating factor

(GM-CSF) and interferon-gamma secretion into the supernatants of aqueous stimulatory T cell cultures were conducted using the above-cloned T cells to illustrate stimulation. Cytotoxicity against macrophages pulsed with the same stimulatory peptide or whole mycobacteria was also assayed. Details of these studies are provided in the Materials and Methods Section. The results are shown in Table 7, below.

Table 7

Protein 540 Peptide-Induced

Stimulatory Responses In T Cell Clones[1]

T cell clones[2]	IL-2	GM-CSF[4]	IFN-Gamma[5]	Cytotoxicity[6]
K8AH + APC	<0.2	11±12 (9%)	10	ND[7]
K8AH + APC + Peptide 24	9.4±0.6	153±30 (>100%)	56	86.5±0.6
K8AH + APC + M. tuberculosis	7.6±0.2	87±35 (71%)	44	85.8±2.9
A7JM + APC	0.2	5±8 (4%)	5±1	ND
A7JM APC + Peptide 22	6.8±0.6	135±4 (>100%)	63±7	82.7±10
A7JM + APC + M. tuberculosis	6.8±0.1	160±14 (>100%)	40	84.7±3

[1] T cell clones were stimulated by the peptide antigen shown hereinbefore to specifically activate each

clone. Stimulation was assayed by four methods. T cells and antigen-presenting cells (APC) without antigen were used as negative controls in all assays. Results are expressed as the mean ± standard deviation (where calculated) of duplicate or triplicate cultures.

[2] Culture contents in addition to the medium are shown in each entry, with plus signs (+) indicating the presence of mixed cellular components and antigen (peptide or M. tuberculosis), where present.

[3] Results expressed in units per ml.

[4] Assay based on three independent studies using three different bone marrow donors. Results are expressed in colony-forming units of GM per $2x10^5$ cells. Parenthesized percentages relate to the number of colonies induced by a GM-CSF positive control supernatant.

[5] Results expressed international units per ml.

[6] Results expressed as percentages as discussed in the Materials and Methods Section. APC + antigen was used as a negative control.

[7] ND = not done.

The results of Table 7 illustrate further standard techniques that are useful in determining the presence of stimulated T cells in addition to the proliferation assays discussed before.

Assays of T cell clones K8AH and A7JM indicated that both showed the helper/inducer ($T4^+$,$T8^-$) phenotype. Cells of the $T4^+$ phenotype are primarily helper cells that recognize antigen plus class II HLA proteins. Such cells are also known to exhibit cytotoxicity as is shown in Table 7.

Tuberculosis is a disease in which the cellular portion of the immune response is involved to the substantial exclusion of the humoral (antibody) portion of the immune response. Thus, the

ability of the preferred peptide antigens to stimulate the T cell clones to not only proliferate but to also secrete IL-2, GM-CSF, and interferon gamma, each of which constitutes a portion of the cellular immunity response, indicates that those peptides, their polymers, and mixtures thereof, as well as the 540 protein (65 KD protein) can play an important role in protective immunity.

That role in cellular immunity is underscored by the macrophage cytotoxicity exhibited by the clones stimulated by the peptides or the whole mycobacteria. Similar cytotoxicity for other mycobacteria-reactive T cell clones has been reported. [Mustafa et al., (1987), Clin. Exp. Immunol., 69:255-262; Kaufman et al. (1986), Lep. Rev. 57, Suppl. 2:101-111.] However, it is believed that the above results are the first demonstration that the same sequence of one protein antigen are involved in both T cell help and cytotoxicity. the *in vivo* role of such $T4^+$ cytotoxic T cells is believed to destroy those macrophages that have become incompetent to kill their intracellularly-growing mycobacteria.

A preferred peptide was previously described herein as being capable of stimulting mycobacterially-immune mononuclear cells, and such a peptide was said to be useful in assaying for present or prior immunological exposure of such cells to mycobacteria. A particularly preferred peptide or its polymer is also capable of immunizing an animal for protection against mycobacterial infection such as *M. tuberculosis*.

Thus, the present invention also contemplates a vaccine against mycobacteria such as *M. tuberculosis* that comprises a physiologically tolerable diluent containing as immunogen an

immunizing effective amount of (i) a peptide whose amino acid residue sequence corresponds substantially to a particularly preferred T cell-stimulating peptide described herein or (ii) a polymer of such a particularly preferred T cell stimulting peptide as described herein.

Exemplary particularly preferred peptides include Peptides 22 and 24. Further particularly preferred peptides are those whose sequences correspond substantially to a sequence of the M. tuberculosis 540 protein or another mycobacterial 65 KD protein and contain 5 to about 40, more preferably about 10 to about 20 residues, and still further are capable of stimulating proliferation of mycobacterially-immune, and for a tuberculosis vaccine, M. tuberculosis-immune, T cells that exhibit the $T4^+$ and/or $T8^+$ phenotype.

Further particularly preferred peptides can be obtained by following a procedure similar to that discussed previously. Polyclonal T cells from one or more individals are contacted with an inoculum containing a peptide such as one of those of Tables 2 and 4, and more particularly peptides such as those of Tables 5 and 6 that have already been shown capable of stimulating proliferation of mycobacterially-immune T cells. The peptides inducing proliferation are noted and the proliferating T cells are cloned by the limiting dilution technique as described by Oftung et al.,. (1987) J. Immunol., 138:927-931. The phenotypes of the proliferated T cells are determined as with the OKT series of monoclonal antibodies available from Ortho Diagnostic Systems, Inc. of Raritan, NJ. One or more of the peptides capable of causing proliferation of T cells having the $T4^+$ and/or $T8^+$ phenotype is utilized in the vaccine.

More preferably, a mixture of peptides, polymers having such peptides as repeating units, or a polymer whose repeating units are a mixture of such peptides that cause proliferation of $T4^+$ and/or $T8^+$ T cells is used. The reason of this preference stems from the already noted MHC restriction. In addition, there is usually found an MHC restriction between $T4^+$ and $T8^+$ T cells, the former recognizing antigen plus class II MHC protein and the latter recognizing antigen plus class I MHC protein.

Peptides that correspond substantially to portions of the 517 protein are also useful herein, and are defined as to substantial correspondence similarly to those peptides discussed before. The peptides substantially corresponding to a sequence of the 517 protein can contain as few as five residues and are therefore somewhat shorter than are the shortest of the peptides discussed before.

Three peptides (denominated 55, 56 and 57) and their variants substantially correspond to sequences, written from left to right in the direction from amino-terminus to carboxy-terminus and using one letter code, having the formulas

55) N N N I G,
56) X G N Z G, and
57) F N S G S G N I G F(I) G N S G

wherein X is an amino acid residue selected from the group consisting of F, S, T, L, D and T; Z is an amino acid residue selected from the group consisting of T, I, L, S and V; and the parenthesized residue can replace the residue shown to its left in the sequence. Thus, in peptide 57, F and I are alternative residues. More preferably, X is selected from the group consisting of F, S and T; and Z is selected from the group consisting of T and I.

Using the before-described consensus matrix to calculate whether the variant pentapeptides defined hereinbefore by the consensus sequence XGNZG correspond substantially, one finds that all of those variants correspond substantially at least at 99% confidence level. This can be readily seen by determining the greatest differences caused by substitutions, then calculating the resultant consensus matrix score, and comparing that value to 3 times the number of residues compared, 5, (3x15=15).

Thus, for the X residue, substituting an Ile (I) for an Asp (D) residue, or a Ser (S) for a Phe (F) provides a value of -3 from the matrix. Similarly for Z, substitution of Ile (I) for Ser (S) or Ser (S) for Val (V) provides a value of -2 from the matrix. Since two Gly (G) residues and the Asn (N) residues are present in any of the before compared consensus pentapeptide sequences, the presence of those residues provides a score of 22 (8+6+8=22). Subtraction of five [(-3)+(-2)] for the above substitutions from 22 provides a total score for the compared pentapeptides of 17.

Since 17 is greater than 15, any of the above substitutions to the consensus sequence provides pentapeptides that correspond substantially at least at the 99% confidence level. Furthermore, since the above substitutions caused the greatest numerical difference in the total score, any other of the before-discussed substitutions for both X and Z in the consensus sequence produces a total score; i.e., where X is Thr or Leu and Z is Thr or Leu, in the consensus sequence produces a total score that is larger than 17, and consequently, all of those pentapeptides also correspond substantially to each other at least at the 99% confidence level.

Peptides 55 and 56 are typically utilized as one of a plurality of repeating units of a polymer having a relatively low molecular weight; i.e., less than about 10,000 daltons in weight. The smallest such polymer, or oligomer, contains two of the five residue peptides (pentapeptides) bonded together through a peptide bond formed between the carboxy-terminal residue of a first pentapeptide repeating unit and the amino-terminal residue of a second pentapeptide repeating unit.

For example, Peptide 57, above, can be viewed as a polymer or oligomer having two such pentapeptide repeating units bonded together by a peptide bond, and also containing an additional four residues at the amino-terminus of the oligomer.

Similar calculations can also be carried out for variants of the other peptides disclosed herein as one means of determining whether a peptide with a different sequence from one of those specifically enumerated corresponds substantially to a specifically enumerated peptide, or to a portion thereof. For the purposes of epitope-paratope interactions, sequences containing at least five residues are the shortest sequences that should be compared since at least five or six residues appear to be required for epitope-paratope interaction. See for example, Elder et al. (1987) J. Virol. 61:8-15; Atassi (1978) Immunochemistry 12:423-438; and Benjamini et al. (1969) Biochemistry 8:2242-2246.

Similarly, the sequence in isolated form

N N N I G N N N I G N N N I G

that is also present at nucelotide positions 3270 through 3226 of Figure 2 can be considered a polymeric or oligomeric trimer of the sequence of

peptide 55. Likewise, an isolated form of the sequence from nucleotide position 3210 through position 3107 can be viewed a polymer or oligomer that contains eight XGNZG pentapeptides repeated. Each of above polymers or oligomers contains a plurality of the pentapeptide repeating units bonded together by peptide bonds.

Solid phase peptide synthesis techniques, as are described in the before-discussed U.S. Patents whose disclosures are incorporated herein by reference, are typically the most useful means of preparation for oligomers and polymers containing up to a total of about forty total residues (eight repeating pentapeptide units).

Genetic engineering techniques as are described herein are particularly useful for preparing larger polymers that contain more than about eight pentapeptide repeating units. For example, a double stranded DNA molecule having the sequence shown in Figure 2 from nucleotide position 2959 through nucleotide position 3303, and in phase with the illustrated amino acid residue sequence of protein 517 can be excised from the larger molecule shown in Figure 2 or synthesized from appropriate deoxyribonucleic acid derivatives using known techniques, and thereafter ligated into an appropriate plasmid vector for expressing a peptide polymer that corresponds substantially in sequence to the polymer containing the pentapeptide repeating units shown beneath the sequence at those positions in Figure 2.

Higher molecular weight polymers; i.e., with average molecular weights of about 10,000 to 1,000,000, or more, containing one or more of the above 540 protein or 517 protein pentapeptide repeating units can also be prepared by oxidatively

polymerizing a peptide that is terminated with cysteine (Cys; C) residues, or a "diCys-terminated" peptide. The resulting polymer thereby contains its repeating units bonded together by oxidized cysteine (cystine) disulfide bonds.

For example, each of the before-discussed 540 protein peptides or 517 protein pentapeptides can be synthesized to contain an additional Cys residue at each of the amino- and carboxy-termini to provide diCys-terminated peptides in their reduced forms. After synthesis, in a typical laboratory preparation, 10 milligrams of the diCys peptide (containing cysteine residues in un-oxidized form) are dissolved in 250 milliliters (ml) of 0.1 molar (M) ammonium bicarbonate buffer. The dissolved diCys-terminated peptide is then air oxidized by stirring the resulting solution gently for period of about 18 hours in the air, or until there is no detectable free mercaptan by the Ellman test. [See Ellman, Arch. Biochem. Biophys., 82:70-77 (1959).]

The polymer so prepared contains a plurality of the synthetic, peptide repeating units that are bonded together by oxidized cysteine (cystine) residues. Such polymers typically contain their peptide repeating units bonded together in a head-to-tail manner as well as in head-to-head and tail-to-tail manners; i.e., the amino-termini of two peptide repeating units can be bonded together through a single cystine residue as can two carboxyl-termini since the linking groups at both peptide termini are identical.

A 517 protein pentapeptide repeating unit can itself be contained in the form of an oligomer containing up to about eight pentapeptide repeating units, or in a shorter peptide such as the 14-residue Peptide 57. Still further, a genetically engineered

polypeptide such as that prepared from the DNA sequence of nucleotides at positions 2959 through 3303 that has been further engineered to include codons for Cys (TGT or TGC) at the 5'- and 3'-ends can also be polymerized.

The molecular weight of such a polymer can be controlled through the addition of chain-terminating reagents. Exemplary chain terminating reagents are cysteine itself and a peptide such as a before-described pentapeptide that further includes a single Cys residue, preferably at a terminus.

The full names for individual amino acid residues are sometimes used herein as are the well-known three letter abbreviations. One letter abbreviations (code) is also utilized. The Table of Correspondence, below, provides the full name as well as the three letter and one letter abbreviations for each amino acid residue named herein (See, for example, L. Stryer, Biochemistry, 2nd ed., W. H. Freeman and Company, San Francisco, (1981), page 16). The amino acid residues utilized herein are in the natural, L, form unless otherwise stated.

Table of Correspondence

Amino acid	Three letter abbreviation	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H

Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

III. MATERIALS AND METHODS

A. Recombinant studies

1. Bacteria, Phage and Plasmids

The E. coli strains used in this work were SNN97 [Young et al., (1983) Science, 222:778-782; ATCC 37194]; JM83 [Yanisch-Perron et al., (1985), Gene, 33:103-119; also ATCC 35607]; JM101 [Yanisch-Perron et al., (1985), Gene, 33:103-119; also ATCC 33876]; Y1089 [Young et al., (1983), Science, 222:778-782; also ATCC 37196]; and Y1090 [Young et al., (1983), Science, 222:778-782; also ATCC 37197]. Plasmids pUC19 [Yanisch-Perron et al., (1985), Gene, 33:103-119] and pMC1871 [Shapira et al., (1983), Gene, 25:71-82] were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. The recombinant DNA library of M. tuberculosis genomic DNA fragments in the λgt11 vector was constructed by R. Young et al. (1985), Proc. Natl. Acad. Sci. USA, 82:2583-2587, and made available through the World Health Organization's Program for Research in the Immunology of Tuberculosis. Recombinant phage λRY3143 and λRY3146 were generously provided by R.A. Young [Whitehead Institute, M.I.T.; Young et al., (1985), Proc. Natl. Acad. Sci. USA,

82:2583-2587]. Subclones of the mycobacterial DNA inserts in these recombinant phage were constructed in pUC19 or M13mp9 [Messing et al., (1982), Gene, 19:269-276; M13mp9 is listed for sale in the August, 1983 catalog of Bethesda Research Laboratories, Inc.] vectors using standard recombinant DNA techniques [Maniatis et al., (1982), Molecular Cloning - a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY].

2. Antisera

Monoclonal antibodies specific for the 65KD antigen were obtained from the Immunology of Tuberculosis Scientific Working Group under a grant from the WHO/World Bank/UNDP Special Program for Vaccine Development. These antibodies included IT-13 [WTB-78; Coates et al., (1981), Lancet, 2:167-169]; IT-31 [SA2D5H4; T. Buchanon, unpublished] and IT-33 [MLIIH9; Gillis et al., (1982), Infect. Immun., 37:172-178]. Anti-beta-galactosidase antibodies were purchased from Cooperbiomedical, Malvern, PA. Polyclonal rabbit antisera directed against a sonicate of M. tuberculosis strain H37Rv were elicited as previously described [Minden et al., (1984), Infect. Immun., 46:519-525].

3. Immunoscreening of λgt11-M. tuberculosis Library

Clones reactive with the monoclonal antibodies specific for the 65KD antigen were isolated essentially as described by Young et al. [Young et al., Proc. Natl. Acad. Sci. USA, 82:2583-2587]. Briefly, for each 150 mm LB plate, 0.6 ml of a fresh overnight culture of Y1090 cells were infected with 1-2x10^5 plaque-forming units (pfu) of the library. After 3.5-4 hours of growth at 42°C, the plaques were overlaid with a dry nitrocellulose filter that had been saturated with 10

millimolar (mM) isopropyl-beta-D-thiogalactopyranoside (IPTG; available from Sigma Chemical Co.). The plates were incubated an additional 3.5-4 hours at 37°C and then removed to room temperature and the position of the filters marked.

The filters were washed briefly in TBST [50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20 [polyoxyethylene (20) sorbitan monolaurate]] and then incubated in TBST plus 20% fetal calf serum. After 30 minutes at room temperature, the filters were transferred to TBST plus antibody.

For the initial screen, the antibody mix contained a 1:1000 dilution of admixed IT-13, IT-31, and IT-33. The filters were incubated with the antibody solution overnight at 4°C with gentle agitation, washed in TBST and reacted with biotinylated goat anti-mouse immunoglobulin, the Vectastain ABC reagent, and developer as described by the manufacturer (Vector Laboratories, Burlingame, CA). After the color had developed, the filters were washed with several changes of water and air dried.

Phage corresponding to positive signals were twice plaque-purified. To determine which monoclonal antibodies reacted with which of the recombinant phage, about 100 pfu of a purified phage stock were inoculated in a small spot on a lawn of Y1090 E. coli on an LB (Luria-Bertani broth) plate. The phage were allowed to grow and induced to synthesize the foreign proteins as described above. The filters were then reacted with a 1:1000 dilution of one of the monoclonal antibodies. The subsequent steps were the same as for the initial screen.

4. Western Blot Assays

Cells containing phage or plasmids in which the expression of the foreign sequences was under the control of the E. coli lac gene regulatory sequences

were induced to synthesize the foreign proteins by incubating the cells in the presence of 2.5 mM IPTG for 2 hours. Crude lysates of cells expressing λgt11 recombinants were made as described in Huynh et al; (1985), DNA Cloning Techniques: A Practical; Gover, ed., IRL Press, Oxford, Vol. I, pp. 49-78. Briefly, those lysates were made by harvesting cells from overnight cultures and resuspending the cells in 10 mM Tris pH 7.5, 10 mM EDTA containing 100 ug lysozyme/ml. After 10 minutes at room temperature, sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%. A protease inhibitor (Trasylol, Boehringer Mannheim, Indianapolis, IN) was added to all crude lysates at a final concentration of 0.03%-0.3%.

The crude protein preparations were electrophoresed on 10% polyacrylamide-SDS Laemmli gels [Laemmli, (1970) Nature, 227:680-685], and the separated proteins electrophoretically transfered to nitrocellulose [Towbin et al., (1979), Proc. Natl. Acad. Sci. USA, 76:4350-4354]. The immobilized proteins were reacted with a 1:1000 dilution of monoclonal antibody IT-13 in TBST overnight at 4°C. The nitrocellulose filters were then washed, reacted with peroxidase-conjugated goat anti-mouse immunoglobulin, and developed as previously described [Niman et al., (1983), Proc. Natl. Acad. Sci. USA, 80:4949-4953].

5. Nucleic Acid Sequencing

The sequences of 5'-end-labeled restriction fragments of the mycobacterial DNA were determined by a modification of the partial chemical degradation technique of Maxam and Gilbert [Brow et al., (1985), Mol. Biol. Evol., 2:1-12; and Maxam et al., (1976), Proc. Natl. Acad. Sci. USA, 74:560-564]. For the M13/dideoxy sequencing studies, Sau3AI fragments from

the mycobacterial DNA inserts were subcloned into the BamHI site of M13mp9. Phage DNA was isolated from the M13 recombinants and subjected to the dideoxy chain termination sequencing reactions [Biggin et al., (1983), Proc. Natl. Acad. Sci. USA, 80:3963-3965; and Sanger et al., (1980), J. Mol. Biol., 143:161-178]. The products of the sequencing reactions were electrophoresed on 6% acrylamide/7M urea/0.5-2.5xTBE gradient sequencing gels, [Biggin, (1983), Proc. Natl. Acad. Sci. USA, 80:3963-3965]. The gels were dried under vacuum and exposed to Kodak XRP-1 film. The nucleotide sequences were determined independantly for both strands of the mycobacterial DNA.

Computer-aided analyses of the nucleic acid sequences and deduced protein sequences were performed using the databases and programs provided by the Nucleic Acid and Protein Identification Resources of the National Institutes of Health as well as the programs of Chow et al., (1978) Adv. Enzym., 47:45-148 and Hopp and Woods [Hopp et al., (1981), Proc. Natl. Acad. Sci. USA, 78:3824-3828].

6. Beta-galactosidase assays

Cells were grown in B broth or B broth plus 2.5 mM IPTG to an optical density at 600 nanometers (OD_{600}) of about 0.3. Crude lysates were made, and beta-galactosidase was activity assayed as described by Miller (1972), Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

7. Capacity of Recombinants to Elicit DCH

a. DCH Assays

Studies were carried out to determine whether the recombinant proteins or purified protein derivative (PPd) (Connaught Laboratories, Ltd., Willowdale, Canada) would elicit DCH reactions in

Hartley guinea pigs that had been immunized with sonicates of M. tuberculosis, M. bovis or saline. Groups of guinea pigs were given three weekly intramuscular (i.m.) injections of sonicates suspended in incomplete Freund's adjuvant (IFA) as the physiologically tolerable diluent. Each injection contained 1.0 milligram (mg) of protein. Some animals received a fourth injection so that one week after the final injection, all animals were tested intradermally (i.d). Test antigens included the crude and partially purified recombinant extracts as well as saline and PPD as controls. Test antigens were used at 1-10 ug diluted in 100 ul of phosphate-buffered saline at a pH value of pH 7.0 (PBS), containing 0.0005% Tween 20 as the physiologically tolerable diluent. Groups of unimmunized guinea pigs were similarly tested. All i.d. injections were administered into shaved areas on guinea pig flanks. Reactions were read at 24, 48 and 72 hours, and were considered positive when the diameters of erythema and indurated areas exceeded 10 mm.

b. Preparation of Crude Lysates

E. coli containing a plasmid or lambda phage of interest were grown by incubation at 37 degrees C with aeration in B-broth to late phase in which absorbance at 600 nanometers (A_{600}) was between approximately 0.4 and 0.6. IPTG was then added to a final concentration of 10 mM and the bacteria were further incubated for two hours.

The bacterial culture was then chilled on ice for 10 minutes and the cells were harvested by centrifugation at 6000 rpm for 10 minutes. The resulting cell pellet was washed once in TBS (50 mM Tris, pH 8, 150 mM NaCl) by resuspension and recentrifiguation, and was thereafter resuspended

(Sigma Chemical Co., St. Louis, MO) in a volume of TBS with 0.5 molar sucrose equivalent to 1/10 the original culture volume. Lysozyme was added to the resulting resuspended solution to a final concentration of 50 ug/ml, and that admixture was incubated for 10 minutes at 37 degrees C. Cells were harvested by centrifugation and were resuspended in an equal volume of TBS. Thereafter, DNAse, Trasylol and SDS (Sigma) were added to the resulting admixture such that the final concentrations were 1 ug/ml, 0.1% and 1%, respectively. That admixture was further incubated at room temperature for a time period of 10 minutes with periodic mixing to effect completion of cell lysis. The resulting crude lysate was stored at -20 degrees C until use.

c. Partial Purification of Expressed 65KD Protein

Proteins containing the 65KD antigens were partially purified from crude lysates of E. coli expressing that protein by differential ammonium sulfate precipitation. To that end, a crude lysate was first combined with a solution of saturated ammonium sulfate (SAS) to give a final concentration of 30% of the original lysate concentration. Precipitation was effected as is well known in the art, and the resulting supernate was retained. The supernate was then combined with SAS to give a concentration of 50% of that of the original lysate, and precipitation effected again. The resulting pellet was retained, resuspended in PBS and dialysed against PBS. This resulting dialysed material is referred to as partially purified.

d. Preparation of Extracts of M. tuberculosis

M. tuberculosis strain H37Rv and M. bovis strain BCG were obtained from the culture collection

of the National Jewish Hospital and Research Center, Denver, CO, and grown as previously described [Minden et al., (1972) Science, 176:57-58 and Minden et al., (1972) Infect. Immun., 6:574-582].

Bacteria were then heat-killed and broken by sonication with ultrasonic treatment until, by microscopic examination, greater than 95% of the cells were disrupted. These disrupted bacteria were then subjected to ultracentrifugation at 200,000xg for a time period of 2 hours, and the supernate was retained. The supernates so obtained are referred to as H37Rv-S and BCG-S, repectively, and their antigenic and biological characteristics have been described.

B. Peptide Studies

1. Mycobacterial antigens

Armadillo derived killed M. lepraе was supplied by Dr. R. J. W. Rees, Mill Hill in London, from the IMMLEP (WHO) bank. M. tuberculosis and M. bovis BCG were kindly donated by Dr. Eng, National Institute of Public Health, Oslo, Norway. Bacilli were killed by irradiation (2.5 m rad). Recombinant M. tuberculosis 65 KD antigen, expressed from λgt11 as a beta-galactosidase fusion protein, were purified from E. coli lysates prepared as described in Oftung et al., (1987) J. Immunol., 138:927-931 on a high affinity anti-beta-galactosidase column (Promega Biotech, Madison, USA).

2. Synthetic peptides

The protected peptide resins were prepared by usual Merrifield solid phase techniques in groups of 100 by the method of Simultaneous Multiple Peptide Synthesis [Houghten, (1985) Proc. Natl. Acad. Sci. USA, 82:5131-5135; and Houghten et al., (1985) Inter. J. Pept. Prot. Res., 27:673-678], and were cleaved twenty-four at a time in a new multi-vessel apparatus

[Houghten et al., (1986) Biotechniques, 4:522-529]. Each synthesis resulted in the generation of 50-75 mg of peptide. Typical purities of the crude peptides ranged from 65-95%.

3. T-cell clones and lines

The T-cell clone K8AH from a tuberculosis patient (AH) and the T-cell clone A7JM from a killed M. leprae-vaccinated person (JM) were established by the limiting dilution technique as described [Oftung et al., (1987) J. Immunol., 138:927-931]. The T-cell line was raised from peripheral blood mononuclear cells (PBMC) of the donor JM by stimulation of $2x10^6$ PBMC/ml in complete medium (RPMI 1640 + 15% AB serum + 1% penicillin and streptomycin) with M. bovis BCG (20 ug/ml) in 24-well Costar plates. After 6 days of incubation at 37°C in an atmosphere of 5% CO_2 and 95% air, antigen-reactive cells were expanded by adding 100 U/ml recombinant IL-2 two times per week. After long term storage in liquid nitrogen, T cells were propagated in vitro by stimulation of $2x10^5$ cells/ml in 24 well Costar plates with whole bacilli as antigen (20 ug/ml) in the presence of 10^6 irradiated autologous PMBC as feeder (antigen-presenting) cells and recombinant IL-2 (100 U/ml). Efficient expansion of clones and lines was achieved by stimulation with antigen and feeder cells once and IL-2 twice per week. Determination of T cell subsets was performed as previously described [Oftung et al., (1987) J. Immunol., 138:927-931].

4. Peptide-Induced T Cell Clone Stimulation Assays

The following assays were carried out for the inventors by Dr. Frederik Oftung, at the Laboratory for Immunology, Norwegian Radium Hospital, Oslo, Norway. Initial assays for T cell stimulation were carried out using coded samples.

a. Antigen-Induced Proliferation of T-cell Clones and Lines

Clonal ($1x10^4$) or polyclonal ($2x10^4$) T cells and irradiated autologous PBMC ($1x10^5$) were distributed to each well of 96-well flat bottom Costar plates. Mycobacterial antigens as whole bacilli, recombinant antigens as affinity-purified material or synthetic peptides were then added in triplicates or duplicates. The total culture volume was kept at 200 microliters (ul).

After 72 hours of incubation, the cultures were given a 4 hour pulse of 0.04$ mBq [^{3}H]-thymidine (specific activity = $185x10^3$ mBq/mM). Cells were then harvested and radioactivity incorporated was determined by liquid scintillation counting [Mustafa et al., (1983) Clin. Exp. Immunol., 52:29-37].

The results are expressed as mean (triplicates or duplicates) values of counts per minute (cpm). Cells were considered to be proliferating in response to a given antigen where cpm in cultures with antigen minus cpm in cultures without antigen was more than 1000 and cpm in cultures with antigen divided by cpm in cultures without antigen was more than 2.

b. Lymphokine Production and Assay

T-cell clones ($2x10^5$ cells/ml) were distributed to wells of 24-well Costar plates with adherent cells from $1x10^6$ irradiated autologous PBMC plus antigen at optimal concentrations. Cell free supernatants were collected after 16 or 48 hours of incubation and stored at minus 20°C until assayed for lymphokine activities. IL-2 activity in supernatants harvested after 16 hours of incubation was determined by their ability to stimulate an IL-2-dependent mouse T-cell clone (CTLL 2) to

proliferate [Mustafa et al., (1985) Clin. Exp. Immunol., 62:474-481]. Granulocyte macrophage-CSF (GM-CSF) activity in the same supernatants was assayed by the capacity of the supernatants to induce colony formation in mononuclear bone marrow cells [Dahl et al., (1972) Acta Pathol. Microbiol. Scand. Sect. B, 80:863-870]. Supernatants harvested after 48 hours were used to determine interferon-gamma activity by the method of Dahl and Degree [Acta Pathol. Microbiol. Scand. Sect. B, 80:863-870], using human embryonic lung fibroblasts and vesicular stomatitis virus as the challenge virus.

c. Cytotoxicity assay

Adherent cells from $1x10^6$ autologous irradiated PBMC in 24-well Costar plates were pulsed with antigens at optimal concentrations and the density of T cell clones was adjusted to $1x10^5$ cells/well. After 7 days of incubation, T cells were washed off, and 0.5 ml of 0.03% neutral red (in saline + 10% FBS) were added to each well and the plates incubated for 30 minutes. Neutral red was then removed from the wells by washing, and the dye taken up by macrophages was released by adding 0.5 ml of 0.05 M acetic acid in 50% ethanol [Parish et al., (1983) J. Immunol. Methods, 58:225]. Percentage cytotoxicity was calculated from spectrophotometric absorbance measurement at 540 nanometers [OD_{540}] according to the formula:

$$\text{Cytotoxicity (\%)} = \frac{OD_{540}\text{con.} - OD_{540}\text{ study}}{OD_{540}\text{ con.}}$$

where OD_{540} con. = OD_{540} of control cultures with adherent cells + T-cell clones; and OD_{540} study = OD_{540} of study cultures with adherent cells + T-cell clones + antigen.

5. Peptide-Induced Pooled T Cell Stimulation

Stimulation assays of pooled human T cells were carried out for the inventors by Dr. Stefan Kaufman of the Max Plank Institute for Immunology, Freiberg, West Germany. Again, coded samples were supplied for the assays.

The assay procedure was as follows. Mononuclear cells were isolated from peripheral blood of M. bovis BCG-vaccinated persons on Ficoll-Hypaque gradients [Emmerich et al., (1986) J. Exp. Med., 163:1024-1029; and Boyum, (1968) Scand. J. Clin. Lab. Invest., 21 (Suppl. 97):31], and were used to seed wells of a 96-well microtiter plate at about $2x10^5$ cells/well. Antigen was then added at 0.1 ug/ml, 1 ug/ml and 10 ug/ml.

After six days of culture, 1 microCurie (uCi) of [^{3}H]-thymidine was added to each well. Eighteen hours later, cultures were harvested on glass fiber filters. Thymidine incorporation was measured in a liquid scintillation counter.

For the assays of Table 5, the PBMC were pooled. For the studies conducted related to HLA restrictions, the PBMC were kept separate and the HLA alleles ascertained by standard techniques.

The present invention has been described with respect to preferred embodiments. It will be clear to those skilled in the art that modifications and/or variations of the disclosed subject matter can be made without departing from the scope of the invention set forth herein.

WHAT IS CLAIMED IS:

1. An isolated DNA molecule that consists essentially of the nucleotide sequence, from right to left and in the direction from 5'-end to 3'-end, that corresponds substantially to the sequence represented by the formula in Figure 2 from about position 3950 to about position 2390, and in a consistent reading frame coding for a 517 amino acid residue protein of Mycobacterium tuberculosis.

2. The DNA molecule of claim 1 having the sequence that consists essentially of the nucleotide sequence, from right to left and in the direction from 5'-end to 3'-end, corresponding to the sequence represented by the formula in Figure 2 from position 3948 through position 2398.

3. A plasmid vector comprising a replicon operationally linked to a foreign DNA sequence that corresponds substantially to a DNA molecule of claim 1, said vector being capable of replicating said foreign DNA sequence in a replication/expression medium.

4. The plasmid vector of claim 3 wherein said replication/expression medium is a unicellular organism.

5. The plasmid vector of claim 3 further including sequence-encoded signals for initiation and termination of transcription that are operationally linked to the 5'-end and the 3'-end, respectively, of said foreign DNA sequence and compatible with said replication/expression medium for transcribing a product coded for by said foreign DNA sequence and expressing a protein product coded for by said DNA sequence.

6. The protein produced by expression of the protein coded for by said foreign DNA of said plasmid vector of claim 5.

7. A bacterial culture comprising bacteria that contain the plasmid vector of claim 5 in an aqueous medium appropriate for the expression of the 517 amino acid residue protein of Mycobacterium tuberculosis.

8. A method of producing a 517 amino acid residue protein of Mycobacterium tuberculosis comprising the steps of:

(a) culturing a replication/expression medium containing a plasmid vector for replicating and expressing a foreign DNA sequence contained therein, said vector containing a foreign DNA sequence corresponding substantially to the DNA molecule of claim 1, said vector additionally containing operatively linked nucleotide sequences regulating replication and expression of said foreign DNA sequence, said culturing being carried out under conditions suitable for expression of the protein encoded by said foreign DNA sequence; and

(b) harvesting the expressed protein that is encoded by said DNA sequence.

9. The method of claim 10 wherein said replication/expression medium is a culture of unicellular organisms.

10. A method for determining previous immunological exposure, of a mammalian host to Mycobacterium tuberculosis or Mycobacterium bovis comprising the steps of:

(a) administering intradermally to an assayed mammalian host an inoculum that consists essentially of the purified 540 amino acid residue protein encoded for by the DNA sequence of Figure 2 or an immunologically active portion thereof, said protein dissolved or dispersed in a physiologically tolerable diluent and present in said diluent in an

amount effective to induce erythema and induration in a mammalian host previously immunized with M. tuberculosis or M. bovis;

(b) maintaining said mammal for a time period of about 24 to about 72 hours; and

(c) assaying for the presence of erythema and induration at the site of intradermal administration at the end of said time period.

11. The method of claim 10 wherein said purified protein is a recombinant protein.

12. The method of claim 11 wherein said purified protein is a recombinant fusion protein that contains a portion of a beta-galactosidase molecule bonded to the amino-terminus of an immunologically active portion of said 540 amino acid residue protein.

13. An inoculum consisting essentially of the purified 540 amino acid residue antigen coded for by the sequence of Figure 2 or an immunologically active portion thereof dissolved or dispersed in a physiologically tolerable diluent and present in said diluent in an amount effective to induce erythema and induration in a mammalian host previously immunized with M. tuberculosis or M. bovis.

14. The inoculum of claim 13 wherein said protein antigen is a recombinant protein.

15. The inoculum of claim 14 wherein said recombinant protein further includes a portion of the beta-galactosidase molecule bonded to the amino-terminus of an immunologically active portion of said 540 amino acid residue protein.

16. A peptide that consists essentially of a 5 to about 40 amino acid residue sequence that corresponds substantially to a sequence of the 540 amino acid residue protein or the 517 amino acid

residue protein coded for by the DNA sequence of Figure 2.

17. The peptide of claim 16 wherein said sequence corresponds substantially to the sequence of the 540 amino acid residue protein represented by a formula written from left to right and in the direction from amino-terminus to carboxy-terminus, selected from the group consisting of

A R R G L E R G L N A L A D A V K V;
E K I G A E L V K E V A K K;
G L K R G I E K A V E K V T E T L;
Q S I G D L I A E A M D K V G N E G V;
L L V S S K V S T V K D L L P;
I E D A V R N A K A A V E E G;
V K V T R S A L Q N A A S I A;
M A K T I A Y D E E A R R G L;
A R R G L E R G L N A L A D A;
A L A D A V K V T L G P K G R;
G P K G R N V V L E K K N G A;
K K N G A P T I T N D G V S I;
D G V S I A K E I E L E D P Y;
L E D P Y E K I G A E L V K E;
E L V K E V A K K T D D V A G;
D D V A G D G T T T A T V L A;
A T V L A Q A L V R E G L R N;
E G L R N V A A G A N P L G L;
N P L G L K R G I E K A V E K;
K A V E K V T E T L L K G A K;
L K G A K E V E T K E Q I A A;
E Q I A A T A A I S A G D Q S;
A G D Q S I G D L I A E A M D;
A E A M D K V G N E G V I T V;
G V I T V E E S N T F G L Q L;
F G L Q L E L T E G M R F D K;

M R F D K G Y I S G Y F V T D;
Y F V T D P E R Q E A V L E D;
A V L E D P Y I L L V S S K V;
L L P L L E K V I G A G K P L;
A G K P L L I I A E D V E G E;
D V E G E A L S T L V V N K I;
V V N K I R G T F K S V A V K;
S V A V K A P G F G D R R K A;
D R R K A M L Q D M A I L T G;
A I L T G G Q V I S E E V G L;
E E V G L T L E N A D L S L L;
D L S L L G K A R K V V V T K;
V V V T K D E T T I V E G A G;
V E G A G D T D A I A G R V A;
A G R V A Q I R Q E I E N S D;
I E N S D S D Y D R E K L Q E;
E K L Q E R L A K L A G G V A;
A G G V A V I K A G A A T E V;
A A T E V E L K E R K N R I E;
K N R I E D A V R N A K A A V;
A K A A V E E G I V A G G G V;
A G G G V T L L Q A A P T L D;
A P T L D E L K L E G D E A T;
G D E A T G A N I V K V A L E;
K V A L E A P L K Q I A F N S;
I A F N S G L E P G V V A E K;
V V A E K V R N L P A G H G L;
A G H G L N A Q T G V Y E D L;
V Y E D L L A A G V A D P V K;
A D P V K V T R S A L Q N A A;
L Q N A A S I A G L F L T T E;
P L T T E A V V A D K P E K E;
K P E K E K A S V P G G G D M; and
K A S V P G G G D M G G M D F.

18. The peptide of claim 16 wherein said sequence corresponds substantially to the sequence of the 540 amino acid residue protein represented by a formula written from left to right and in the direction from amino-terminus to carboxy-terminus, selected from the group consisting of

A V L E D P Y I L L V S S K V;
L L V S S K V S T V K D L L P;
L L P L L E K V I G A G K P L;
A I L T G G Q V I S E E V G L;
I A F N S G L E P G V V A E K;
A R R G L E R G L N A L A D A V K V;
E K I G A E L V K E V A K K;
G L K R G I E K A V E K V T E T L; and
I E D A V R N A K A A V E E G.

19. The peptide of claim 16 wherein said sequence corresponds substantially to the sequence of the 517 amino acid residue protein represented by a formula written from left to right and in the direction from amino-terminus to carboxy-termminus, selected from the group consisting of:

N N N I G,
X G N Z G, and
F N S G S G N I G F(I) G N S G

wherein X is an amino acid residue selected from the group consisting of F, S, T, L , D and I; Z is an amino acid residue selected from the group consisting of T, I, L, S and V; and the parenthesized residue can replace the residue shown to its left in the sequence.

20. A polymer comprising a plurality of peptide repeating units, said peptide repeating units consisting essentially of an amino acid residue sequence that corresponds substantially to a sequence of a 65KD mycobacterial cell wall protein-a, said peptide repeating units having the capacity of stimulating T cells immune to the mycobacteria of said 65KD mycobacterial cell wall protein-a.

21. The polymer of claim 20 wherein said mycobacterium is M. tuberculosis or M. bovis.

22. The polymer of claim 21 wherein said peptide repeating units consist essentially of a sequence, written from left to right and in the direction from amino-terminus to carboxy-terminus, represented by a formula selected from the group consisting of

A V L E D P Y I L L V S S K V;
L L V S S K V S T V K D L L P;
L L P L L E K V I G A G K P L;
A I L T G G Q V I S E E V G L;
I A F N S G L E P G V V A E K;
A R R G L E R G L N A L A D A V K V;
E K I G A E L V K E V A K K;
G L K R G I E K A V E K V T E T L; and
I E D A V R N A K A A V E E G.

23. The polymer of claim 22 wherein said peptide repeating units are bonded together by oxidized cysteine residues present at the termini of said repeating units.

24. The polymer of claim 20 wherein said mycobacterium is M. leprae, and said peptide repeating units consist essentially of a sequence, written from left to right and in the direction from

amino-terminus to carboxy-terminus, represented by a formula selected from the group consisting of

A V L E E P Y I L L V S S K V; and
I A P N S G M E P G V V E K.

25. A polymer comprising a plurality of pentapeptide repeating units, each of said pentapeptide repeating units consisting essentially of a sequence, written from left to right and in the direction from amino-terminus to carboxy-terminus, represented by the formula

N N N I G; or
X G N Z G,

wherein X is an amino acid residue selected from the group consisting of F, S, T, L, D and I; and Z is an amino acid residue selected from the group consisting of T, I, L, S and V.

26. The polymer of claim 25 wherein said pentapeptide repeating units are bonded together by oxidized cysteine residues present at the termini of said repeating units.

27. A method for assaying for the presence of an infection of M. tuberculosis in a patient comprising the steps of:

a) providing a solid phase support comprised of a 540 amino acid residue protein coded for by the M. tuberculosis genome or an immunologically active portion thereof as antigen affixed to a solid phase matrix;

b) admixing a liquid sample from a patient with said solid phase support to form a solid-liquid phase admixture;

c) maintaining said admixture for a time period sufficient for antibodies to said 540 amino acid residue protein present in said sample to bind to said antigen of said solid support;

d) separating the solid and liquid phases; and

e) determining the presence of antibodies bound to the solid phase support.

28. The method of claim 27 wherein said antigen is a recombinant protein.

29. The method of claim 28 wherein said recombinant protein is a fusion protein that further includes a portion of the beta-galactosidase molecule bonded to the amino-terminus of said antigen.

30. A diagnostic kit comprising a package that contains a solid support comprised of a purified 540 amino acid residue protein encoded by the M. tuberculosis genome as antigen affixed to a solid phase matrix.

31. The diagnostic kit of claim 30 further including a second package that contains a labeled reagent that reacts with human antibodies bound to said solid support.

32. A method for ascertaining the presence of mycobacterially-immune mammalian mononuclear cells in a body sample comprising the steps of

(a) admixing and contacting mammalian mononuclear cells to be assayed in an aqueous medium with a stimulating amount of both antigen-presenting cells and a mycobacterial antigen to form a stimulatory cell culture, said mycobacterial antigen comprising

(i) a 65KD cell wall protein-a of said mycobacteria,

(ii) a recombinant fusion protein containing an immunologically active portion of said 65KD protein, or

(iii) a peptide consisting essentially of a sequence of 5 to about 40 amino acid residues that correspond substantially to a sequence of the 65KD cell wall protein-a of said mycobacteria and is capable of stimulating mycobacterially-immune T cells;

(b) maintaining said stimulatory cell culture for a time period sufficient for mycobacterially-immune mononuclear cells present to be stimulated and to evidence stimulation; and

(c) determining the presence of mononuclear cell stimulation.

33. The method of claim 32 wherein said mycobacterially-immune mononuclear cells are immune to either M. tuberculosis or M. bovis, and said mycobacterial antigen is a peptide antigen that has a sequence of about 10 to about 30 amino acid residues.

34. The method of claim 33 wherein said peptide antigen has a sequence, written from right to left and in the direction from amino-terminus to carboxy-terminus, represented by a formula selected from the group consisting of

A V L E D P Y I L L V S S K V;
L L V S S K V S T V K D L L P;
L L P L L E K V I G A G K P L;
A I L T G G Q V I S E E V G L;
I A F N S G L E P G V V A E K;
A R R G L E R G L N A L A D A V K V;
E K I G A E L V K E V A K K;
G L K R G I E K A V E K V T E T L; and
I E D A V R N A K A A V E E G.

35. The method of claim 34 wherein said method is carried out in vivo, said antigen-presenting cells are provided endogenously and said aqueous culture medium is endogenous blood or lymph.

36. The method of claim 34 wherein said method is carried out in vitro, and said stimulation is determined by an assay selected from the group consisting of (i) mononuclear cell proliferation, (ii) interleukin-2 secretion, (iii) interferon-gamma secretion, (iv) granulocyte macrophage-colony stimulating factor secretion and (v) cytotoxicity.

37. The method of claim 34 wherein said peptide antigen is present as a polymer having repeating units comprised of said peptide antigen, and said peptide antigen repeating units are bonded together by oxidized cysteine residues at the termini thereof.

38. A diagnostic assay kit comprising a container that includes a mycobacterial antigen present in an amount sufficient to carry out at least one assay, said mycobacterial antigen comprising

(i) a peptide antigen,

(ii) a polymer of said peptide antigen repeating units in which said peptide antigen consists essentially of a sequence of 5 to about 40 amino acid residues that correspond substantially to a sequence of the 540 amino acid residue protein coded for by the DNA sequence of Figure 2 and is capable of stimulating mycobacterially-immune T cells,

(iii) a fusion protein that includes an immunologically active portion of said 65KD cell wall protein-a.

39. The diagnostic assay kit of claim 38 wherein said mycobacterial antigen is a peptide antigen or a polymer of said peptide antigen repeating units, and said peptide antigen and said peptide antigen polymer repeating units have an amino acid residue sequence, written from right to left and from amino-terminus to carboxy-terminus, represented by a formula selected from the group consisting of

A V L E D P Y I L L V S S K V;
L L V S S K V S T V K D L L P;
L L P L L E K V I G A G K P L;
A I L T G G Q V I S E E V G L;
I A F N S G L E P G V V A E K;
A R R G L E R G L N A L A D A V K V;
E K I G A E L V K E V A K K;
G L K R G I E K A V E K V T E T L; and
I E D A V R N A K A A V E E G.

40. A vaccine against mycobacterial infection comprising a physiologically tolerable diluent and an immunizing amount of (i) a peptide containing a sequence of 5 to about 40 amino acid residues whose amino acid residue sequence corresponds substantially to a sequence of a mycobacterial 65KD cell wall protein-a and that is capable of stimulating T cells immune to said mycobacterium that have a phenotype selected from the group consisting of $T4^+$ and $T8^+$, or (ii) a polymer having said peptide antigen as repeating units.

41. The vaccine of claim 40 wherein said mycobacterium is M. tuberculosis and said peptide antigen or said peptide antigen repeating units contain about 10 to about 20 amino acid repeating units.

42. The vaccine of claim 41 wherein said peptide has a sequence, written from left to right and in the direction from amino-terminus to carboxy-terminus, represented by the formula

A V L E D P Y I L L V S S K V; and
L L P L L E K I G A G K P L.

43. Paratopic molecules that immunoreact with a peptide containing 5 to about 40 amino acid residues that corresponds substantially in sequence to the 540 amino residue protein coded for by the DNA sequence of Figure 2 and also to said 540 amino acid residue protein.

44. The paratopic molecules of claim 43 wherein said peptide has an amino acid residue sequence, written from right to left and in the direction from amino-terminus to carboxy-terminus, represented by a formula selected from the group consisting of

M A K T I A Y D E E A R R G L; and
K A S V P G G G D M G G M D F.

FIG. 1

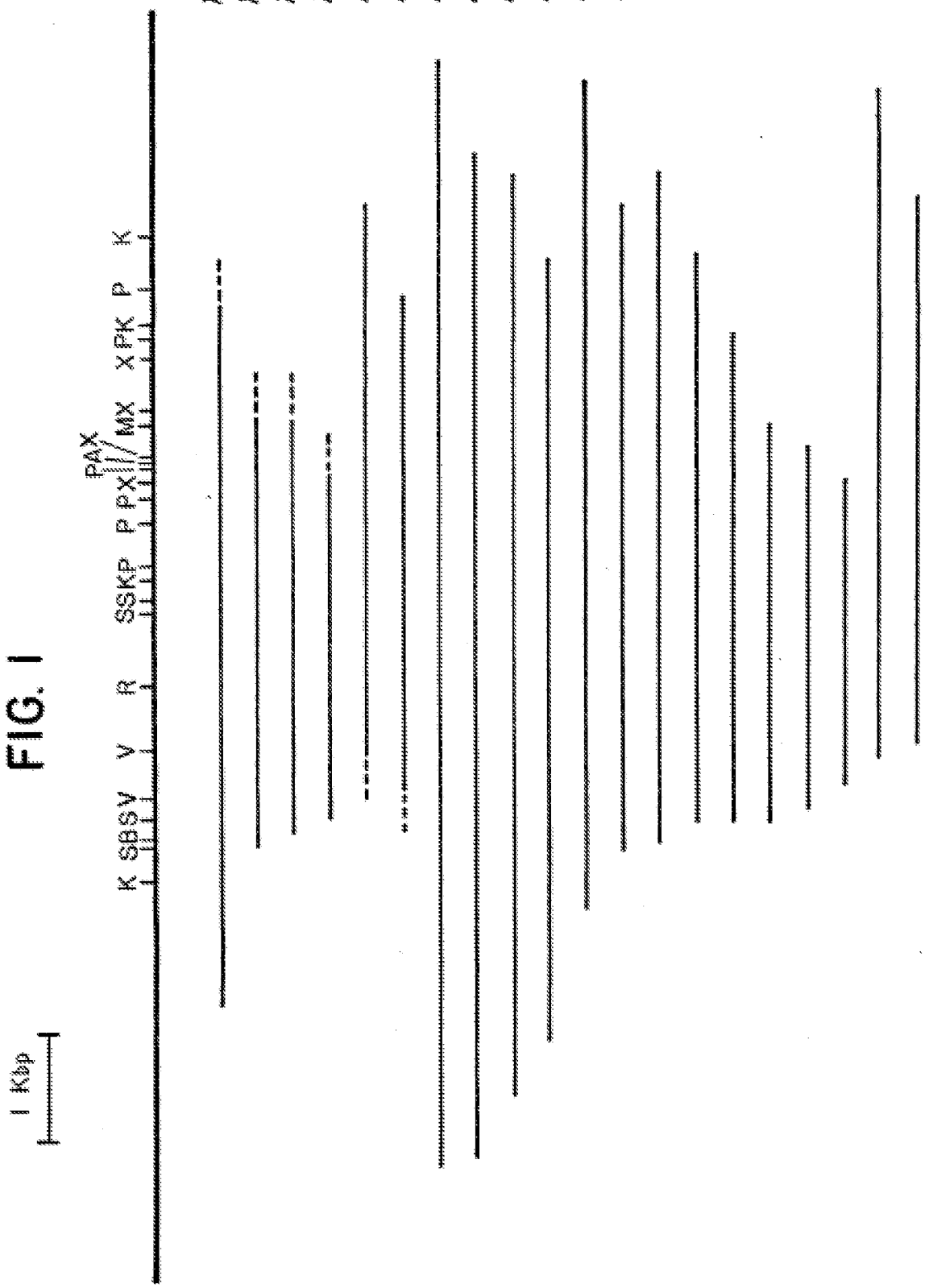

1 Kbp
K SBSV V R SSKP P PX PAX MX X PK P K
λSK119
λSK138
λSK7
λSK14
λSK116
λSK48
λSK63
λSK62
λSK34
λSK121
λSK43
λSK36
λSK120
λSK114
λSK28
λRY3143
λRY3146
λSK17
λSK35
λSK27

FIG. 2a

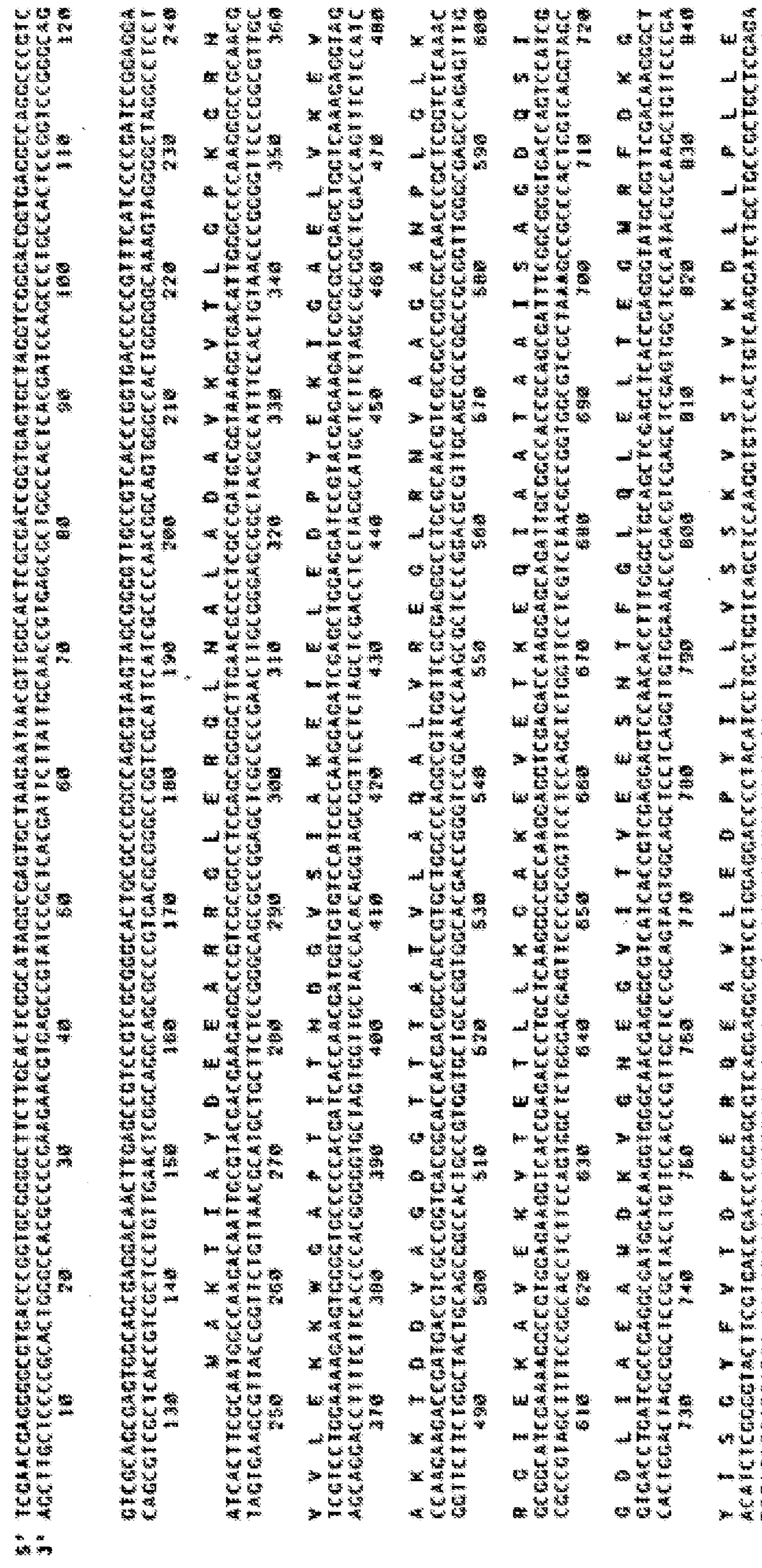


FIG. 2b

FIG. 2c

FIG. 2d

FIG. 3

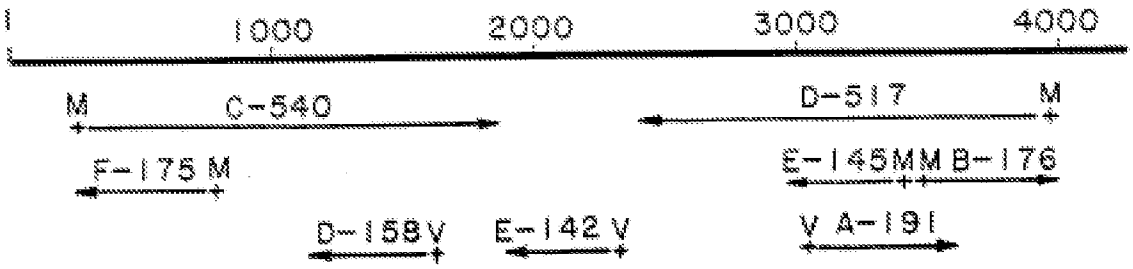
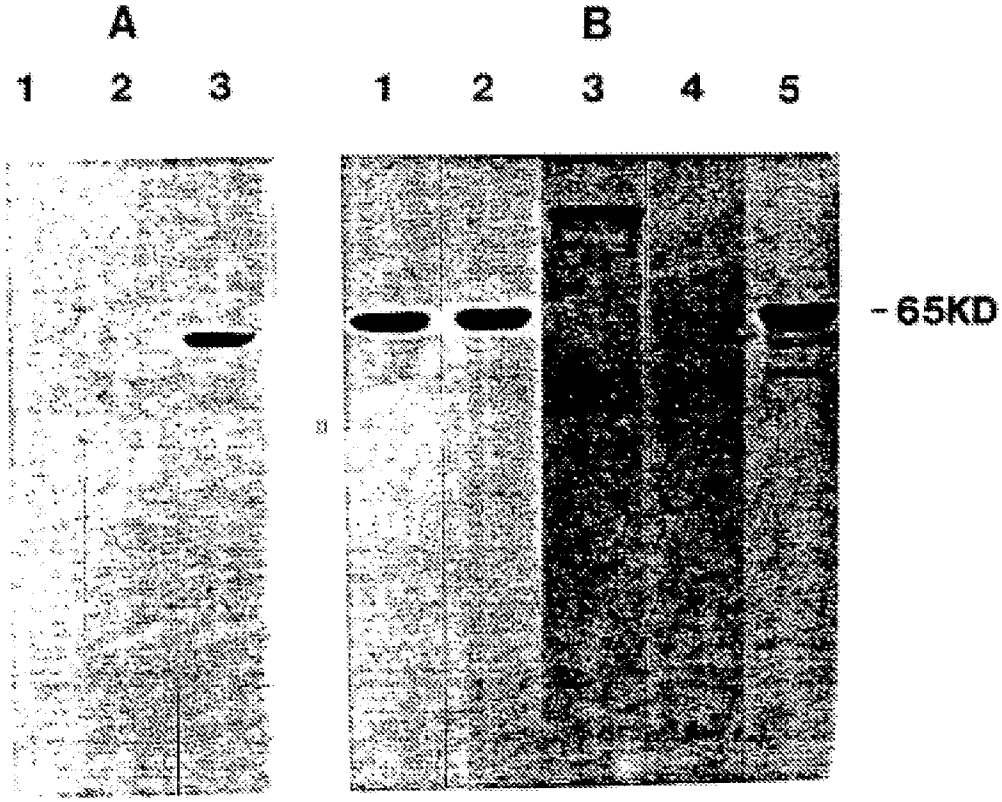

1
1000
2000
3000
4000
M
C-540
D-517
M
F-175 M
E-145MM B-176
D-158V
E-142 V
V A-191

FIG. 4


A
B
1 2 3
1 2 3 4 5
-65KD

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/00598

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4) C07H 15/12; C07K 15/00; A61K 39/04; G01N 33/53
US. CL. 536/27; 530/350; 424/92; 435/7

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	536/27; 530/350; 424/92; 435/7

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CHEMICAL ABSTRACTS AND BIOSIS ABSTRACTS ON LINE COMPUTER SEARCH

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Proceeding National Academy of Science, U.S.A., (Washington, D.C., U.S.A.), Volume 82, issued May 1985 (YOUNG ET AL), "Dissection of Mycobacterium tuberculosis Antigens using Recombinant DNA" see pages 2583-2587.	1-9
A,P	European Journal of Immunology, (New York, USA), Volume 17, No. 3, issued March 1987, (KAUFMANN ET AL), "Enumeration of T cells Reactive with Mycobacterium tuberculosis Organisms and Specific for the Recombinant Mycobacterial 64-KDa Protein". See pages 351-357.	10-15 and 40-42
A,P	Infection and Immunity, (Washington, D.C., USA), Volume 55, No. 10, issued October 1987, (LU ET AL), "Genes for Immunodominant Protein Antigens are Highly Homologous in Mycobacterium tuberculosis, Mycobacterium africanum, and the Vaccine Strain Mycobacterium Bovis BCG. See pages 2378-2382.	16-25 and 43-44

* Special categories of cited documents: [14]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 MAY 1988	28 JUN 1988
International Searching Authority: ISA/US	Signature of Authorized Officer: Abdel A. Mohamed; ABDEL A. MOHAMED

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
A	US,A, 4,489,158, STRAUS PUBLISHED, 18 December 1984 (See columns 1-3 and 16).	27-39
A	US,A, 4,575,484, STRAUS PUBLISHED, 11 March 1986 (See columns 16-18).	27-39

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [1]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-9 are drawn to gene for Mycobacterium tuberculosis with plasmid vector and bacterial culture, classified in class 536, subclass 27. (SEE ATTACHMENT).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. TELEPHONE PRACTICE

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ATTACHMENT TO FORM PCT/ISA/210, PART VI.1

II. Claims 10-15 and 40-42 are directed to vaccine and inoculation for Mycobacterium tuberculosis or Mycobacterium bovis, classified in class 424 and subclass 92.

III. Claims 16-26 and 43-44 are drawn to protein from Mycobacterium tuberculosis gene and peptide fragment of proteins, classified in class 530 subclass 350.

IV Claims 27-39 are directed to Immunoassay and Diagnostic Kit for Mycobacterium tuberculosis, classified in class 435 and subclass 7.